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FOR THE DEGREE OF MASTER OF SCIENCE

# Phylogenetic Relationships of Green Seaweeds, *Ulva* and *Enteromorpha* (Family Ulvaceae) Inferred from nrDNA Internal Transcribed Spacer2 Sequences



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# 국문초록

갈파래과(Ulvaceae)에 속하는 파래속(*Enteromorpha*)과 갈파래속(*Ulva*)은 전세계적으로 광범위하게 분포하며 환경적인 영향을 많이 주고 받는 속이다. 이들은 형태학적으로 속간의 차이가 뚜렷하다고 하지만 환경적 요인에 의하여 변이가 많은 집단으로서 유전자적 수준에서 분류학적인 검증이 필요한 종들이다.

파래속과 갈파래속의 계통분류학적 연구를 위하여 복제한 ITS2 염기서열과 NCBI에 등록된 genebank의 ITS2를 비교 분석하였다. 염기서열 자료를 통한 계통학적 유연관계 분석은 유전적 거리(genetic distance)와 단순성(parsimony)에 근거한 방법을 이용하였다. 재료의 채집은 제주도 다섯 지역에서 시행되었으며 갈파래과(Ulvaceae)의 두 속(*Enteromorpha*, *Ulva*)에 속하는 해조를 4종 11개체 채집하였다.

이번 갈파래과(Ulvaceae)에 대한 연구에서 ITS2의 염기길이는 167에서 203 bp(base pair)로 밝혀졌다. 연구 결과, 파래와 갈파래속은 모든 계통수에서 단진화군(monophyletic group)인 것으로 밝혀졌다. 하지만 형태학적인 특징에 따라 갈파래속과 파래속으로 대표되는 종들은 계통수에서 전형적인 clade가 나타나지 않았다.

이 연구에서 모란갈파래와 구멍갈파래는 두 종간의 형태학적 차이에도 불구하고 단일 clade에 속하는 것으로 밝혀졌다. 또한 파래속과 갈파래, 두 속간에는 뚜렷한 진화적 차이를 나타내지 않으며, 한 식물체에서 엽상형과 관상형 모두를 가질 수 있다는 가능성을 나타냈다.

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# I. Introduction

*Ulva* and *Enteromorpha* are two of the best known marine green algal genera (Tan et al., 1999). They are common inhabitants of the upper intertidal zone of shore, estuaries, and a variety of man-made structures throughout the world (Callow et al., 1997). Tolerance of a wide range of salinities and water qualities, together with the production of large numbers of propagules, contribute to the ecological success of this cosmopolitan genus and to its significance as the most widespread and troublesome ship-fouling macroalga (Callow, 1986). In addition, they are widely used as model organisms for experimental studies of marine biofilms and spore adhesion, plant physiology, as bioindicators of organic and inorganic pollution (Tan et al., 1999).

Linnaeus (1753) published his *Species plantarum* in which he grouped all thalloid algae together as *Ulva*. He recognized nine species in this genus though some of these species are now placed in other related genera and others in unrelated genera. In the 1800s, *Ulva* was re-organized on the basis of gross morphology into three genera: *Ulva*, *Enteromorpha* and *Monostroma* or *Ulvaria* (Woolcott and King, 1993). A species list of marine benethic algae from Korea has been published and they listed four genera and fourteen species in Ulvaceae (Lee et al., 1986; Lee and Kang, 1986).

The members of the family Ulvaceae are tubular or membranous: in the latter case they pass through a tubular state. Many of the species have a diplobiontic, isomorphic life cycle, while a few are entirely asexual. All reproduce by means of biflagellate or quadriflagellate zoospores. All are anchored to the substrate by a basal disc or by rhizoidal filaments that are capable of regenerating new plants (Bold, 1985). *Ulva* and *Enteromorpha* are widely regarded as easily recognizable seaweed genera. *Ulva* species are flat, lettucelike blades with two cell layers thickness, and *Enteromorpha* species form hollow liquid or gas filled tubes with one cell thickness, which may also be highly branched (Tan et al., 1999). However, the genus *Ulva* species are difficult to distinguish from one another on the basis of morphological and

cytological criteria alone because most of these vary with age, season, environmental conditions and geographical location, even within a population at a given time (Coat et al, 1998). And it is also known that intraspecific morphological variation can partly be environmentally induced in the genus *Enteromorpha* species (Leskinen and Pamilo., 1997).

Nuclear ribosomal DNA (nrDNA) Internal Transcribed Spacer2 (ITS2) sequences are known to evolve quickly and have been reported to be useful for the study of intraspecific and interspecific variation and biogeography in algae (Bakker et al., 1992; Kooistra et al., 1992). The ITS sequences are located between the 18S and 28S ribosomal RNA genes, and the region includes the 5.8S rRNA gene and the spacers ITS1 and ITS2 (Baldwin and Johnson, 1993). With analyzing of ITS sequence, Blomster et al. (1998) concluded that *Enteromorpha intestinalis* and *Enteromorpha compressa* represent two distinct, genetically divergent, and reproductively isolated species that happen to be very difficult to distinguish from each other and could be regarded as cryptic species. Blomster et al. (1999) suggested that *Enteromorpha muscoides* (Clemente y rubio) Cremased and *Enteromorpha clatharta* Roth (Greville) are conspecific, with the old name *Enteromorpha muscoides* taking priority based on ITS1 and ITS2 and the 5.8S gene. Also, Malta et al. (1999) proposed that *Ulva lactuca*, *Ulva rigida* and *Ulva scandinavia* from the Veeres meer are all members of one highly polymorphic species based on the ITS2 region. Also, Tan et al. (1999) demonstrated that two genera, *Ulva* and *Enteromorpha* are not monophyletic and that the characteristic of *Ulva* and *Enteromorpha* morphologies has arisen independently several times throughout the evolutionary diversification of the group.

The author will here describe the basic characteristics of the ITS2 sequences in *Ulva* and *Enteromorpha*.

# II. Materials and Methods

## 1. Sampling

*Ulva* and *Enteromopha* thalli were collected from five sites in Jeju (formerly Cheju) (Fig. 1). Epiphytic algae and the stipes were removed from each individual avoiding cross-contamination, and the materials were desiccated in silica gel (Chase and Hills, 1991) or air-dried, and stored at -80℃. Immediately before DNA extraction, the tissue was rehydrated in distilled water and cleaned. Details of algal specimens used in this study are presented in Table 1.



**Fig. 1. Map of Jeju showing the sites where *Ulva* and *Enteromorpha* thalli were collected in 2001.**

**2. Morphological identification**

Samples were identified on the basis of morphological characters such as habit and details of cell arrangement and organelles (Blomster et al., 1998; Maggs and Ward, 1996; Lee et al., 1986). Details of cell morphology were observed in surface view using 100 ~ 1000× magnification microscope (Vickers Ltd.).

**3. DNA extraction**

Algal materials (50 mg) were ground with a ceramic mortar in liquid nitrogen for 2 min. and then DNA was extracted with DNeasy® Plant Mini Kit (QIAGEN Inc.) according to the manufacturer's protocol. DNA extraction was directly used for PCR experiments. DNA quality was checked on 0.8% TAE (Tris-acetate-EDTA) agarose gels stained with ethidium bromide or determined by measuring the absorbance at 260 nm using a Unicam UV/VIS Spectrometer (Heλios β, Unicam Ltd, UK). The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to 280 nm.



**4. PCR amplification**

The polymerase chain reaction (PCR) was used to amplify the nuclear ribosomal internal transcribed spacer2 (ITS2). Primers complementary to the 3'-end of the 5.8S nrDNA and the 5'-end of the 26S nrDNA were used to amplify the ITS2 region (Fig. 2). Details of primers for ITS2 region were described in Table 2. PCR amplification was performed in a Programmable Thermo Controller (PTC-100, MJ Research Inc.) with an initial denaturation step of 94℃ for 2 min. followed by 29 cycles of 94℃ for 45 s, 55℃ for 1 min. and 72℃ for 1 min. The final step was at 72℃ for 3 min. The reaction volume was 50 ㎕ consisting of 5 ㎕ genomic DNA (0.1-0.3 ㎍), 1 μM of each primers, 1 ㎕ of 100 mM $MgSO_4$, 5 ㎕ of 10×reaction buffer, 1 ㎕ of PCR Nucleotide Mix (containing the sodium salts of dATP, dCTP,

Table 1. *Enteromorpha* and *Ulva* species used in the phylogenetic analyses

| Species name | Code | Site | GenBank accession No. | Collection date or literature source |
|---|---|---|---|---|
| *E. intestinalis* | Eint-ham | Hamdeok | | 8 Apr. 2001 |
| *E. intestinalis* | Eint-ojo | Ojo-ri | | 8 Apr. 2001 |
| *E. linza* | Elin-ojo | Ojo-ri | | 8 Apr. 2001 |
| *E. linza* | Elin-jung | Jungmun | | 7 Apr. 2001 |
| *U. pertusa* | Uper-jo | Jocheon | | 8 Apr. 2001 |
| *U. pertusa* | Uper-ojo | Ojo-ri | | 8 Apr. 2001 |
| *U. pertusa* | Uper-seong | Seongsan | | 8 Apr. 2001 |
| *U. pertusa* | Uper-jung | Jungmun | | 7 Apr. 2001 |
| *U. conglobata* | Ucon-jo | Jocheon | | 8 Apr. 2001 |
| *U. conglobata* | Ucon-ojo | Ojo-ri | | 8 Apr. 2001 |
| *U. conglobata* | Ucon-seong | Seongsan | | 8 Apr. 2001 |
| *E. intestinalis* | Eint-AF202467 | | AF202467 | Blomster et al. (2000) |
| *E. intestinalis* | Eint-AF202468 | | AF202468 | Blomster et al. (2000) |
| *E. prolifera* | Epro-AF035354 | | AF035354 | Blomster et al. (1998) |
| *E. prolifera* | Epro-AJ234304 | | AJ234304 | Tan et al. (1999) |

Table 1. Continued

| Species name | Code | Site | GenBank accession No. | Collection date or literature source |
|---|---|---|---|---|
| *E. linza* | Elin-AJ000204 | | AJ000204 | Tan et al. (1999) |
| *E. linza* | Elin-AJ000203 | | AJ000203 | Tan et al. (1999) |
| *E. linza* | Elin-AF153491 | | AF153491 | Malta et al. (1999) |
| *E. compressa* | Ecom-AF202466 | | AF202466 | Blomster et al. (2000) |
| *E. compressa* | Ecom-AJ234302 | | AJ234302 | Tan et al. (1999) |
| *U. pseudocurvata* | Upse-AJ234312 | | AJ234312 | Tan et al. (1999) |
| *U. rigida* | Urig-AF153490 | | AF153490 | Malta et al. (1999) |
| *U. californica* | Ucal-AJ234315 | | AJ234315 | Tan et al. (1999) |
| *U. lactuca* | Ulac-AJ234311 | | AJ234311 | Tan et al. (1999) |
| *U. lactuca* | Ulac-AJ000208 | | AJ000208 | Tan et al. (1999) |
| *U. fenestrata* | Ufen-AJ234316 | | AJ234316 | Tan et al. (1999) |
| *U. pertusa* | Uper-AJ234321 | | AJ234321 | Tan et al. (1999) |
| *Blidingia minima* | Bmin-AJ000206 | | AJ000206 | Tan et al. (1999) |
| *Monostroma grevillei* | Mgre-AJ000205 | No data | AJ000205 | Tan et al. (2000) |

* Collection sites for Genbank datas refer to literature.

Table 2. Primers used for PCR amplification and sequencing

| Primer Name | Sequence | Target | Direction |
|---|---|---|---|
| 5.8S-F1[a] | 5′GTG AAT TGC AGA ATT CCG TC 3′ | ITS2 | Forwaed |
| 26S[a]-R1 | 5′GCC TCA CCT GAA CTC AGG TC 3′ | ITS2 | Reverse |
| SK[b] | 5′CGC TCT AGA ACT AGT GGA TC 3′ | | |
| T7[b] | 5′GTA ATA CGA CTC ACT ATA GGG C 3′ | | |

a: primers designed for PCR amplification

b: primers designed for the pBluescript phagemid vector based on STRATAGENE



Fig. 2. Location of primer sites for amplification and sequencing of the rDNA ITS2 region in *Ulva* and *Enteromorpha*.

dGTP, and dTTP, each at a concentration of 10 mM in water, Promega Co.), and 0.5 to 1 unit of *Vent* polymerase (New England BioLabs Inc.). Approximately 2 drops of mineral oil from a 200 ㎕ micropipette tip was added prior to the initiation of cycling to serve as an evaporation barrier. To estimate the size of the amplified fragment, the product was run on a 0.8% agarose gel (Agarose LE, Promega Co.) stained with 0.5 ㎍/ml ethidium bromide in 1× TAE buffer with a 100bp DNA ladder (MBI Fermentas), visualized under UV light, and photographed, The presence of a single bright band in each lane of the gel was a check for a successful amplification. The desired DNA fragment was cut out of the agarose gel with a sterile scalpel and purified using the QIAEX Ⅱ Gel Extraction Kit (QIAGEN Inc.) according to the manufacturer's instructions.

**5. Cloning of the PCR product**



For cloning of the PCR products, *E. coli* strains (XL1-Blue MRF' and HB101) and pBluescript II SK(-) were used as hosts and a vector. 2.5 ㎍ of vector pBluescript II SK(-) (STRATAGENE) was digested with the restriction enzyme, Hinc Ⅱ at 37℃ for 2 hrs. The digested vector was purified using a High Pure PCR Product Purification Kit (Roche Molecular Biochemicals) according to the supplier's protocol. After purification, the concentration of purified product was determined on an agarose gel. Ligation was performed in 20 ㎕ reaction mixtures consisting of 1 ㎕ of pBluescript II SK(-) vector (cutted with Hinc Ⅱ), 5 to 10 ㎕ of insert DNA, 4 ㎕ of 5× ligation buffer, and 1 unit of T4 DNA ligase (TAKARA SHUZO CO., LTD). The mixture was incubated at 15℃ for 16 hours. The ligated products mixed with XL1-Blue MRF' competent cells were placed on ice for 40 min. and then heat-shocked for 1.5 min at 42℃. After adding 200 ㎕ of Luria-Bertani (LB) broth, the mixture was incubated for 30 min. at 37℃ to increase the efficiency of transformation.

The *E. coli* cells were spread on Luria-Bertani (LB) agar containing ampicillin, X-gal, and IPTG and incubated overnight at 37℃. White colonies were inoculated

into 4 ml LB broth containing ampicillin and grown overnight at 37℃ with shaking . Plasmid DNA was isolated from *E. coli* following protocol of Molecular Cloning (Sambrook and Rusell, 2001).

**6. Sequencing**

DNA sequencing was performed using an SEQ4X4 personal sequencing system (Amersham Pharmacia Biotech) with a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) according to the supplier's guide.

For each sequencing reaction, 27 ㎕ of the master mix (approximately 600 ng of plasmid DNA; 3.5 ㎕ of reaction buffer (150 mM Tris-HCl, pH 9.5, 35 mM $MgCl_2$); 2 ㎕ of 1 μM sequencing-primer; 2 ㎕ of thermo sequenase DNA polymerase (10 U/㎕); and distilled water to adjust total volume to 27 ㎕) was prepared in a microcentrifuge tube. After the contents of the master mix were mixed thoroughly, 7 ㎕ of the master mix was aliquoted into each tube (labelled A, C, G, and T) containing 1 ㎕ of the corresponding Cy5.5 ddNTP termination mix. After each sequencing reaction was mixed thoroughly, one drop of mineral oil from a 200 ㎕ micropipette tip was added to each reaction mix. The cycle-sequencing conditions were conducted with the following process: (1) an initial denaturation for 2 min. at 95 ℃ and (2) 29 cycles of 95℃ for 30 s, 60℃ for 30 s, and 72℃ for 1.5 min. For some combinations of primers and templates, higher (60℃) or lower (50℃) annealing temperatures were used to optimize the cycle-sequencing reactions.

For purification of sequencing reactions, the products were purified by ethanol precipitation method according to the manufacturer's guides. DNA sequencing samples were loaded on an automated SEQ4×4 sequencer and the profile was analyzed automatically by the software SEQ4×4 Basecaller in the end of each run.

T7 and SK primers were used to obtain first 5' and 3' end of sequence information, respectively. Sequences were obtained on both strands in ITS2 region.

**7. Data analysis**

For phylogenetic analysis, unalignable sequence data were excluded from the full data set of sequences generated from individual primers. New sequences were aligned with published sequences (Table 1). Initial sequence alignments were constructed using the Clustral X program. Sequences were modified with inspection by eye from the profile of fluorescent peaks. Sequences from GenBank were aligned with those of this study for data analysis (Table 1).

Distance analyses were conducted by the tree-building algorithm of Neighbor-Joining (NJ) (Saitou and Nei, 1987; NJ) and Minimum Evolution (ME) methods with Kimura' two parameters (Kimura, 1980), Jukes-Cantor (Jukes, 1969) and Tamura and Nei's (Tamura and Nei, 1993) distances within the program MEGA (version 2b3) (Kumar et al., 2001), respectively. In the parsimony analysis, the phylogenetic tree was constructed by the Maximum Parsimony (MP) (Fitch, 1971) method with the program PAUP (version 4.0b8) (Swofford, 1998). Base composition and patterns of substitution for pairwise comparisons were also analyzed with MEGA.

Bootstrappings (Felsenstein, 1985) of 1000 replications were performed to evaluate statistically the strength of support for each internal node in resulting trees. Bootstrap analyses were conducted with MEGA for the NJ and ME methods and with PAUP for the MP method.

All trees were rooted with *Monostroma grevillei* and *Blidingia minima*, which were chosen as the outgroup because they represent a different genus and their sequences were alignable with, but more divergent than, all *Enteromorpha* and *Ulva* sequences.

# III. Results

## 1. ITS2 properties and alignment

In this study for Ulvaceae, the length of ITS2 varied between 167 and 203 bp (Table 3.). These lengths were not comparable to each other genera in this research because the boundaries of the ITS regions are not all coincident. Especially, the length s of *Enteromorpha intestinalis* (AF202467) and *Ulva lactuca* (AJ000208) were shorter than others (Blomster et al., 2000; Tan et al., 1999

The ITS2 sequence alignment used in this study is shown in Fig. 3. Table 3 shows that base compositions of ITS2 is not equal with each other. The result of alignment exhibits that G+C content values were higher than A+T with ranging from 65. 7% to 76.1%, excluding outgroups (G+C : 68.6 ± 2.4%, T : 16.6 ± 1.6%, A : 14.7 ± 1.4% on average).



## 2. Intra- and interspecific divergence

Pairwies divergence in the ITS2 region using the Jukes and Cantor distance for samples of the Ulvaceae ranged from 0% to 26.2%. Considerable variability within individual was detected in *Ulva pertusa* (Talbe 4). There was a low level of sequence divergence between the *U. pertusa* and *U. conglobata* collected in Jeju (from 0% to 1.7%), while *U. pertusa* (AJ234321) showed a high level of divergence (from 12.5% to 14.4%). Also, the level of divergence in *Enteromorpha intestinalis* showed a high rate (6.0%). On the other hand, *E. linza* had a low level of divergence (from 0% to 0.8%). Significantly, the divergence between *U. pseudocurvata* and *E. compressa* showed a low level (0%) even though genus was different. And this group exhibited that the divergence was in excess 17.5% with others. Although *E. intestinalis* and *E. compressa* was known to be similar with morphologically, their degree of divergence

was very high (from 16.4 to 18.5%).

### 3. Phylogenetic analyses

The Phylogenetic trees obtained from all analyses (Fig. 4 ~ Fig. 11), showed various clades of the Ulvaceae. Phylogenetic trees were constructed by distance methods (NJ, ME) and parsimony-based method (MP). MP was analyzed in weighted (Tv:Ts = 3:1) and unweighted. All phylogenetic analyses resulted in a monophyletic two genera *Ulva/Enteromorpha* assemblage with 100% bootstrap support, but the respective genera were not monophyletic (Fig. 4 ~ 11). Sequence divergence within the *Ulva/Enteromorpha* clade ranged from 0% to 26.2%. *Enteromorpha intestinalis* and *Ulva pseudocurvata* , which occupied a strongly supported sister group position to all other *Ulva* and *Enteromorpha* (Fig 4 ~ Fig. 9) exhibited a divergences in excess of 10% with all others. Several strongly supported interspecific and intergenetic clades were evident within which sequence divergence was extremely low. The phylogenetic analyses clearly showed that the overall morphology of a sample was not correlated with its positon within the *Ulva/Enteromorpha* clade (Fig. 4 ~ Fig. 11). These results were comparable with those of Tan et al. (1999).

*Ulva pertusa* and *Ulva congolobata* assemblage collected in Jeju, were strongly supported by bootstrap values (BP = 91%) as a monophyletic group (Fig. 10 and Fig. 11). Sequence divergence within this clade ranged from 0% to 1.7%, showing same levels of sequence divergence within other clearly monospecific groupings. So there was no evolutionary difference between them though *U. pertusa* samples had a perforated morphology, and *U. conglobata* samples had a fasciculate morphology. Also, *U. rigidia* showed that sequence divergence between the members is low (from 0% to 1.7%). However, another sample (Uper-AJ234321) was not grouped in same clade showing a low divergence (from 12.5% to

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Eint-AF202468 ---------- -CCCCT--CA C--CCG---- -----CTCAC GC---GGGTG GACCT [ 55]
Eint-AF202467 ---------- -.....--.. .--...---- -----..... ..---..... ..... [ 55]
Ecom-AJ234302 -----GGATA C.....--.. .GC...---- -----...G. .A---.A.C. .G..G [ 55]
Upse-AJ234312 -----GGATA C.....--.. .GC...---- -----...G. .A---.A.C. .G..G [ 55]
Ecom-AF202466 ---------- -.....--.. .GC...---- -----...G. .A---.A.C. .G..G [ 55]
Ulac-AJ234311 -----GAATA A.....--.. .G--..---- -----..TG. -----.C... ..... [ 55]
Ucal-AJ234315 -----GAATA A.....--.. .G--..---- -----..TG. -----.C... ..... [ 55]
Uper-jung     -----GAATA A.....--.. .G--..---- -----..T-- -----.CTG. -.... [ 55]
Uper-ojo      -----GAATA A.....--.. .G--..---- -----..T-- -----.CTG. -.... [ 55]
Uper-seong    -----GAATA A.....--.. .G--..---- -----..T-- -----.CTG. -.... [ 55]
Ucon-seong    -----GAATA A.....--.. .G--..---- -----..T-- -----.CTG. ..... [ 55]
Ucon-jo       -----GAATA A.....--.. .G--..---- -----..T-- -----.CTG. ..... [ 55]
Ucon-ojo      -----GAATA A.....T-.. .G--..---- -----T.GC- -----.C... ..... [ 55]
Uper-jo       -----GAATA A.....T-.. .G--..---- -----T.GC- -----.C... ..... [ 55]
Urig-AF153490 ---------- A.....--.. .G--.----- -----T.GC- -----.C... ..... [ 55]
Elin-AJ000204 -----GAATA C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Elin-AF153491 ---------- C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Elin-AJ000203 -----GAATA C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Elin-ojo      -----GAATA C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Elin-jung     -----GAATA C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Eint-ojo      -----GAATA C.....--.. .GCA.T---- -----.C.-- -----.C... ..... [ 55]
Eint-ham      -----GAATA C.....--.. .GCA.T---- -----.C.-- -----.C... ..... [ 55]
Epro-AJ234304 -----GAATA C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Epro-AF035354 ---------- C.....--.. .GCA.C---- -----.C.-- -----.C... ..... [ 55]
Uper-AJ234321 -----GAATA C.....CG.G .GG..C---- -----.C.CG C.---.C.C. ..... [ 55]
Ufen-AJ234316 -----GAATA C.....CA.C .GTG..GACA TGCGTG..CG CG---CA... ..G.. [ 55]
Ulac-AJ000208 ---------- ---------- ---------- ---------- ---------- ..A.. [ 55]
Mgre-AJ000205 TAATAGTGCA A.....--.. .TC..CCTGC CCTCG.GGCG .ACGG.A... ..... [ 55]
Bmin-AJ000206 -----GTGAA AA....--.. .-C..TCTCC CCTTG.CGGG AGCGG.C... ..A.. [ 55]
```



Fig. 3. Alignment of the ITS2 sequences of species of *Enteromorpha* and *Ulva*, and *Monostroma grevillei* and *Blidinggia minima* used as outgroup. Dots represent nucleotides identical to those of the first sequence, and dashes indicate gaps.

```
Eint-AF202468 GGCCCCCCCG GC-CGGCCCC TCGCGGGCT- --GGCCGGGC CGGCTG-AAA TACAG [110]
Eint-AF202467 .......... ..-....... .........- --........ ......-... ..... [110]
Ecom-AJ234302 T.GA..T--. ..-.CC...G GTC.TT..-- --........ T.....-..G ..... [110]
Upse-AJ234312 T.GA..T--. ..-.CC...G GTC.TT..-- --........ T.....-..G ..... [110]
Ecom-AF202466 T.GA..T--. ..-.CC...G GTC.TT..-- --........ T.....-..G ..... [110]
Ulac-AJ234311 .......... ..-..C.GG. C.--.T..CG GCA..... T.....-... .G... [110]
Ucal-AJ234315 .......... ..-.AC.GG. C.C-.T..CG GCA..... T.....-... .G... [110]
Uper-jung     .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Uper-ojo      .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Uper-seong    .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Ucon-seong    .......... .T-.ACTGG. C.C..T..C- --A..... ......G... ..... [110]
Ucon-jo       .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Ucon-ojo      .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Uper-jo       .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Urig-AF153490 .......... .T-.ACTGG. C.C..T..C- --A..... ......-... ..... [110]
Elin-AJ000204 .......... .A-..C.T.G G.-------- --.C.... ......-... .G... [110]
Elin-AF153491 .......... .A-..C.T.G G.-------- --.C.... ......-... .G... [110]
Elin-AJ000203 .......... .A-..C.T.G G.-------- --.C.... ......-... .G... [110]
Elin-ojo      .......... .A-..C.T.G G.-------- --.C.... ......-... .GT.. [110]
Elin-jung     .......... .A-..C.T.G G.-------- --.C.... ......-... .GT.. [110]
Eint-ojo      .......... .A-.TC...G ---------- ---T..... T.....-... .G... [110]
Eint-ham      .......... .A-.TC...G ---------- ---T..... ......-... AG... [110]
Epro-AJ234304 .......... .TG.ACTA.G GT-------- --.T..... ......-... ..... [110]
Epro-AF035354 .......... .TG.ACTG.G GT-------- --.T..... ......-... ..... [110]
Uper-AJ234321 .......... .A-..CT..G C.-------- --.C..... ......-... .G... [110]
Ufen-AJ234316 .......... .G-..CGA.. ...------- TC.C..... ......-... .G... [110]
Ulac-AJ000208 .......... .G-..CGA.A ...------- TC.C..... ......-... .G... [110]
Mgre-AJ000205 ..T.T....A T--..CTT.G G..------- ----AT.... T.....-... C..G. [110]
Bmin-AJ000206 ..T.T..T.A .G-.CC.T.G .G.G------ ---T.T.... T.....-... .TTG. [110]
```



Fig. 3. Continued.

```
Eint-AF202468 AGGCT-CGTG CGCGGCCCAT TCGTGGCCCC GACTAGGTAG GTAGCTCGCT ACTTC [165]
Eint-AF202467 .....-.... .......... .......... .......... .......... ..... [165]
Ecom-AJ234302 ...T.-.... .......... ...C...... .......... ..T......A ..... [165]
Upse-AJ234312 ...T.-.... .......... ...C...... .......... ..T......A ..... [165]
Ecom-AF202466 ...T.-.... .......... ...C...... .......... ..T......A ..... [165]
Ulac-AJ234311 .....-.... .......... ...C...... .......... .......... ...G. [165]
Ucal-AJ234315 .....-.... .......... ...C...... .......... .......... ...G. [165]
Uper-jung     .....-..C. .......... ...C...... .......... .......... ...C. [165]
Uper-ojo      .....-..C. .......... ...C...... .......... .......... ...C. [165]
Uper-seong    .....-..C. .......... ...C...... .......... .......... ...C. [165]
Ucon-seong    .....-..C. .......... ...C...... .......... .......... ...C. [165]
Ucon-jo       .....-..C. .......... ...C...... .......... .......... ...C. [165]
Ucon-ojo      ..CG.-..C. .......... ...C...... .......... .......... ...C. [165]
Uper-jo       ..CG.-..C. .......... ...C...... .......... .......... ...C. [165]
Urig-AF153490 .....-..C. .......... ...C...... .......... .......... ...C- [165]
Elin-AJ000204 .....-.... .......... .......... .......... .......... ..... [165]
Elin-AF153491 .....T.... .......... .......... .......... .......... ..... [165]
Elin-AJ000203 .....-.... .......... .......... .......... .......... ..... [165]
Elin-ojo      .....-.... .......... .......... .......... .......... ..... [165]
Elin-jung     .....-.... .......... .......... .......... .......... ..... [165]
Eint-ojo      .....-.... .......... .......... .......... .......... ..... [165]
Eint-ham      .....-.... .......... .......... .......... .......... ...G. [165]
Epro-AJ234304 .....-.... .......... .......... .......... .......... ...G. [165]
Epro-AF035354 .....-.... .......... .......... .......... .......... ...G. [165]
Uper-AJ234321 .....-..C. .........C .......... .......... .......... ...G. [165]
Ufen-AJ234316 .....-..C. .........C .......... .......... .......... ...G. [165]
Ulac-AJ000208 .....-..C. .........C .......... .......... .......... ...G. [165]
Mgre-AJ000205 ..AT.-.AC. .T...G..TG .A-C...AA. AG.A...... ..G.....-- TTCA. [165]
Bmin-AJ000206 ...TG-.AA. .AT..A..TG .A-....AA. AG.A...... ........-- T..A. [165]
```



Fig. 3. Continued.

```
Eint-AF202468 TA-GGCGGCG GCTCGGTG-T CGCGTGCTGT GAGCCCC-AA GGA-ACATC- CTTTC [220]
Eint-AF202467 ..-..... ........-. .......... .......--- ---------- ----- [220]
Ecom-AJ234302 ..-.....A. ........-C ..T.....A. .....A.G.. ...T..TAA- ..... [220]
Upse-AJ234312 ..-.....A. ........-C ..T.....A. .....A.G.. ...T..TAA- ..... [220]
Ecom-AF202466 ..-.....A. ........-C ..T.....A. .....A.G.. ...T..TAA- ..... [220]
Ulac-AJ234311 ..-.....A. ......C.-. .......... .....A.G.G ...T..CCAT .CA.- [220]
Ucal-AJ234315 ..-.....A. ......C.-. .......... .....A.G.G ...T..CCAT .CA.- [220]
Uper-jung     ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Uper-ojo      ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Uper-seong    ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Ucon-seong    ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Ucon-jo       ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Ucon-ojo      ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Uper-jo       ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.- [220]
Urig-AF153490 ..C.....A. ......C.-. .......... .....A.G.. ...T..CCA- ..A.T [220]
Elin-AJ000204 ..-.....T. ........-. .......... .....G...GA. ...T..CAAT .CA.T [220]
Elin-AF153491 ..-.....T. ........C. .......... .....G...GA. ...T..CAAT .CA.T [220]
Elin-AJ000203 ..-.....T. ........-. .......... .....G...GA. ...T..CAAT .CA.T [220]
Elin-ojo      ..-.....T. ........-. .......... .....G....-A. ...T..CAAT .CA.T [220]
Elin-jung     ..-.....T. ........-. .......... .....G....-A. ...T..CAAT .CA.T [220]
Eint-ojo      ..-.....T. ........-. .......... .....G....-G. A..T..CAAT .CA.T [220]
Eint-ham      ..-.....A. ........-. ........T. .....G....-C. ...T..CCAT .CA.T [220]
Epro-AJ234304 ..-.....A. ........-. .......... .....G....GA. ...T.TCCAT .CA.- [220]
Epro-AF035354 ..-.....A. ........-. .......... .....GT...GA. ...T.TCCAT .CA.- [220]
Uper-AJ234321 ..-..... ......C.CC T...C..... .....G.....AG. ...T.ACC.C .CA.- [220]
Ufen-AJ234316 ..-..... ......C.C. .......... .....G....GAC. ...T.ACC.C TCA.- [220]
Ulac-AJ000208 ..-..... ......C.C. .......... .....G....GA.. ...T.ACC.C TCA.T [220]
Mgre-AJ000205 .CC...T.AT ...TA.G.CA ....CA.GA- -.T..T-TC. .A.A..CCTT T-.A. [220]
Bmin-AJ000206 .CCA..T..T ...TT.G.CC .A.T...AAG -C....-GC. ...-----TT TC... [220]
```



Fig. 3. Continued.

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Eint-AF202468 CATTCGACC- ---------- [240]
Eint-AF202467 ---------- ---------- [240]
Ecom-AJ234302 ...------- ---------- [240]
Upse-AJ234312 ...------- ---------- [240]
Ecom-AF202466 .........T GAGTTCAGGT [240]
Ulac-AJ234311 ---------- ---------- [240]
Ucal-AJ234315 ---------- ---------- [240]
Uper-jung     ---------- ---------- [240]
Uper-ojo      ---------- ---------- [240]
Uper-seong    ---------- ---------- [240]
Ucon-seong    ---------- ---------- [240]
Ucon-jo       ---------- ---------- [240]
Ucon-ojo      ---------- ---------- [240]
Uper-jo       ---------- ---------- [240]
Urig-AF153490 .--------- ---------- [240]
Elin-AJ000204 ...------- ---------- [240]
Elin-AF153491 .--------- ---------- [240]
Elin-AJ000203 ...------- ---------- [240]
Elin-ojo      ...------- ---------- [240]
Elin-jung     ...------- ---------- [240]
Eint-ojo      ---------- ---------- [240]
Eint-ham      ---------- ---------- [240]
Epro-AJ234304 ---------- ---------- [240]
Epro-AF035354 ---------- ---------- [240]
Uper-AJ234321 ---------- ---------- [240]
Ufen-AJ234316 ---------- ---------- [240]
Ulac-AJ000208 ...------- ---------- [240]
Mgre-AJ000205 AC.------- ---------- [240]
Bmin-AJ000206 ACC.TT---- ---------- [240]
```

Fig. 3. Continued.

Table 3. Length (n = number of nucleotides) and GC% of ITS2 in *Enteromorpha* and *Ulva*

| Species | ITS 2 | | | | | |
|---|---|---|---|---|---|---|
| | T | C | A | G | GC % | n |
| 1. Eint-AF202468 | 17.3 | 38.2 | 12.6 | 31.9 | 70.1 | 191 |
| 2. Eint-AF202467 | 16.2 | 38.9 | 10.2 | 34.7 | 73.6 | 167 |
| 3. Ecom-AJ234302 | 18.8 | 33.3 | 15.1 | 32.8 | 66.1 | 192 |
| 4. Upse-AJ234312 | 18.8 | 33.3 | 15.1 | 32.8 | 66.1 | 192 |
| 5. Ecom-AF202466 | 19.7 | 33.0 | 14.8 | 32.5 | 65.5 | 203 |
| 6. Ulac-AJ234311 | 15.3 | 36.0 | 14.3 | 34.4 | 70.4 | 189 |
| 7. Ucal-AJ234315 | 15.3 | 36.3 | 14.7 | 33.7 | 70.0 | 190 |
| 8. Uper-jung | 15.7 | 37.3 | 16.2 | 30.8 | 68.1 | 185 |
| 9. Uper-ojo | 15.7 | 37.3 | 16.2 | 30.8 | 68.1 | 185 |
| 10.Uper-seong | 15.7 | 37.3 | 16.2 | 30.8 | 68.1 | 185 |
| 11. Ucon-seong | 15.5 | 36.9 | 16.0 | 31.6 | 68.5 | 187 |
| 12. Ucon-jo | 15.6 | 37.1 | 16.1 | 31.2 | 68.3 | 186 |
| 13. Ucon-ojo | 16.0 | 36.7 | 16.0 | 31.4 | 68.1 | 188 |
| 14. Uper-jo | 16.0 | 36.7 | 16.0 | 31.4 | 68.1 | 188 |
| 15. Urig-AF153490 | 15.9 | 37.9 | 14.8 | 31.3 | 69.2 | 182 |
| 16. Elin-AJ000204 | 17.3 | 36.2 | 15.1 | 31.4 | 67.6 | 185 |
| 17. Elin-AF153491 | 17.2 | 37.8 | 13.3 | 31.7 | 69.5 | 180 |
| 18. Elin-AJ000203 | 17.3 | 36.2 | 15.1 | 31.4 | 67.6 | 185 |
| 19. Elin-ojo | 17.9 | 35.9 | 15.2 | 31.0 | 66.9 | 184 |
| 20. Elin-jung | 17.9 | 35.9 | 15.2 | 31.0 | 66.9 | 184 |
| 21. Eint-ojo | 19.1 | 35.4 | 15.2 | 30.3 | 65.7 | 178 |
| 22. Eint-ham | 17.4 | 37.1 | 15.2 | 30.3 | 67.5 | 178 |
| 23. Epro-AJ234304 | 17.6 | 34.6 | 15.9 | 31.9 | 66.5 | 182 |
| 24. Epro-AF035354 | 18.1 | 35.6 | 14.1 | 32.2 | 67.8 | 177 |
| 25. Uper-AJ234321 | 11.7 | 43.1 | 12.2 | 33.0 | 76.1 | 188 |
| 26. Ufen-AJ234316 | 15.0 | 37.5 | 13.5 | 34.0 | 71.5 | 200 |
| 27. Ulac-AJ000208 | 15.4 | 35.8 | 14.8 | 34.0 | 69.8 | 162 |
| 28. Mgre-AJ000205* | 20.1 | 32.7 | 19.6 | 27.6 | 60.3 | 199 |
| 29. Bmin-AJ000206* | 23.5 | 30.6 | 16.8 | 29.1 | 59.7 | 196 |
| Mean | 17.0 | 36.2 | 15.1 | 31.7 | 67.9 | 185.8 |

All frequencies are given in percent. Outgroups were indicated by asterisks.

Table 4. Divergence matrix of ITS2 sequences showing Jukes and Cantor distances for samples of *Enteromorpha* and *Ulva*. Box indicates *Ulva conglobata* and *Ulva pertusa* collected in this study.

| No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | 17.5 | 17.5 | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | 17.5 | 17.5 | 0 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | 17.5 | 17.5 | 0 | 0 | | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | 8.7 | 8.7 | 17.5 | 17.5 | 17.5 | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 | 9.6 | 9.6 | 17.5 | 17.5 | 17.5 | 8 | | | | | | | | | | | | | | | | | | | | | | | |
| 8 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | | | | | | | | | | | | | | | | | | | | | | |
| 9 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | 0 | | | | | | | | | | | | | | | | | | | | | |
| 10 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | 0 | 0 | | | | | | | | | | | | | | | | | | | | |
| 11 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | 0 | 0 | 0 | | | | | | | | | | | | | | | | | | | |
| 12 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | 0 | 0 | 0 | 0 | | | | | | | | | | | | | | | | | | |
| 13 | 11.5 | 11.5 | 20.6 | 20.6 | 20.6 | 6.9 | 6.0 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | | | | | | | | | | | | | | | | | |
| 14 | 11.5 | 11.5 | 20.6 | 20.6 | 20.6 | 6.9 | 6.0 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 0 | | | | | | | | | | | | | | | | |
| 15 | 9.6 | 9.6 | 19.6 | 19.6 | 19.6 | 5.1 | 4.2 | 0 | 0 | 0 | 0 | 0 | 1.7 | 1.7 | | | | | | | | | | | | | | | |
| 16 | 5.1 | 5.1 | 18.5 | 18.5 | 18.5 | 8.7 | 9.6 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 13.4 | 13.4 | 11.5 | | | | | | | | | | | | | | |
| 17 | 5.1 | 5.1 | 18.5 | 18.5 | 18.5 | 8.7 | 9.6 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 13.4 | 13.4 | 11.5 | 0 | | | | | | | | | | | | | |
| 18 | 5.1 | 5.1 | 18.5 | 18.5 | 18.5 | 8.7 | 9.6 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 13.4 | 13.4 | 11.5 | 0 | 0 | | | | | | | | | | | | |
| 19 | 6.0 | 6.0 | 19.6 | 19.6 | 19.6 | 9.6 | 10.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 14.4 | 14.4 | 12.5 | 0.8 | 0.8 | 0.8 | | | | | | | | | | | |
| 20 | 6.0 | 6.0 | 19.6 | 19.6 | 19.6 | 9.6 | 10.5 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 14.4 | 14.4 | 12.5 | 0.8 | 0.8 | 0.8 | 0 | | | | | | | | | | |
| 21 | 6.0 | 6.0 | 16.4 | 16.4 | 16.4 | 8.7 | 8.7 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 14.4 | 14.4 | 12.5 | 2.5 | 2.5 | 2.5 | 3.4 | 3.4 | | | | | | | | | |
| 22 | 7.8 | 7.8 | 18.5 | 18.5 | 18.5 | 9.6 | 9.6 | 12.5 | 12.5 | 12.5 | 12.5 | 12.5 | 14.4 | 14.4 | 12.5 | 5.1 | 5.1 | 5.1 | 6.0 | 6.0 | 4.2 | | | | | | | | |
| 23 | 6.9 | 6.9 | 18.5 | 18.5 | 18.5 | 9.6 | 8.7 | 7.8 | 7.8 | 7.8 | 7.8 | 7.8 | 9.6 | 9.6 | 7.8 | 5.1 | 5.1 | 5.1 | 6.0 | 6.0 | 6.0 | 5.1 | | | | | | | |
| 24 | 7.8 | 7.8 | 19.6 | 19.6 | 9.6 | 8.7 | 7.8 | 7.8 | 7.8 | 7.8 | 7.8 | 7.8 | 9.6 | 9.6 | 7.8 | 6.0 | 6.0 | 6.0 | 6.9 | 6.9 | 6.9 | 6.0 | 1.7 | | | | | | |
| 25 | 10.5 | 10.5 | 10.5 | 23.9 | 23.9 | 23.9 | 12.5 | 13.4 | 12.5 | 12.5 | 12.5 | 12.5 | 14.4 | 14.4 | 12.5 | 8.7 | 8.7 | 8.7 | 9.6 | 9.6 | 9.6 | 9.6 | 8.7 | 9.6 | | | | | |
| 26 | 8.7 | 8.7 | 26.2 | 26.2 | 26.2 | 9.6 | 10.5 | 10.5 | 10.5 | 10.5 | 10.5 | 10.5 | 12.5 | 12.5 | 10.5 | 7.8 | 7.8 | 7.8 | 8.7 | 8.7 | 9.6 | 9.6 | 7.8 | 9.6 | 6.0 | | | | |
| 27 | 9.6 | 9.6 | 26.2 | 26.2 | 26.2 | 10.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 13.4 | 13.5 | 11.5 | 7.8 | 7.8 | 7.8 | 8.7 | 8.7 | 9.6 | 9.6 | 7.8 | 9.6 | 6.0 | 1.7 | | | |
| 28 | 54.1 | 54.1 | 55.8 | 55.8 | 55.8 | 50.8 | 52.4 | 49.2 | 49.2 | 49.2 | 49.2 | 49.2 | 49.2 | 49.2 | 49.2 | 52.4 | 52.4 | 52.4 | 54.1 | 54.1 | 54.1 | 54.1 | 50.8 | 49.2 | 50.8 | 55.8 | 55.8 | | |
| 29 | 47.6 | 47.6 | 54.1 | 54.1 | 54.1 | 50.8 | 50.8 | 54.1 | 54.1 | 54.1 | 54.1 | 54.1 | 55.8 | 55.8 | 54.1 | 44.6 | 44.6 | 44.6 | 43.1 | 43.1 | 44.6 | 47.6 | 47.6 | 49.2 | 47.6 | 47.6 | 46.1 | 29.8 | |

* No. : Species number listed in table 3. All frequencies are given in percent.

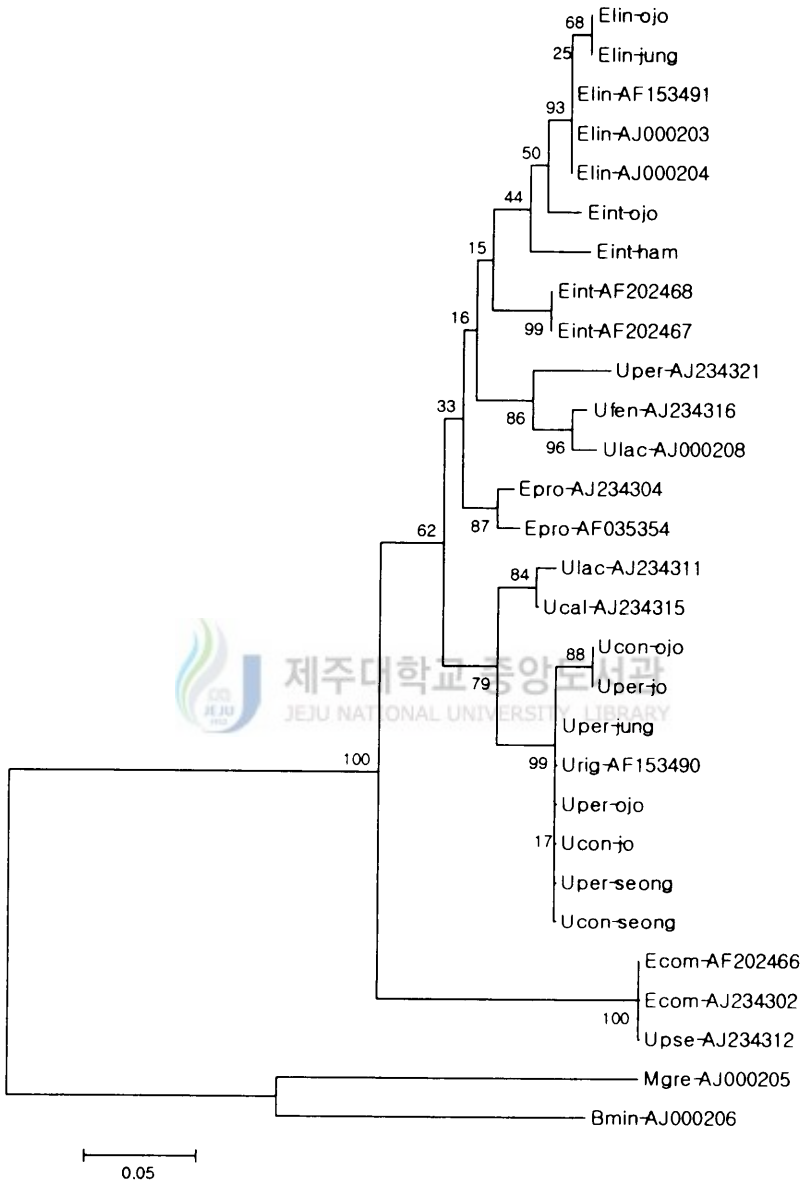



Fig. 4. Bootstrap tree for ITS2 using the NJ method – Jukes and Cantor distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale bar).

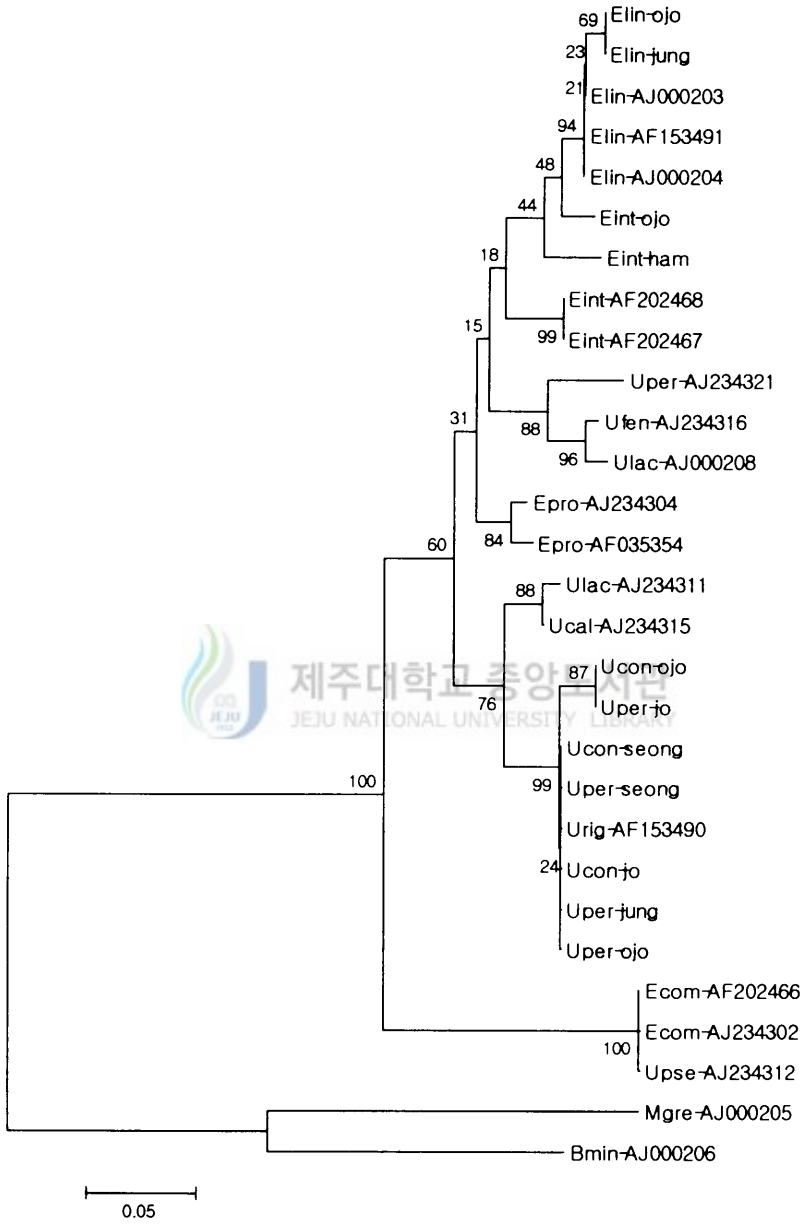


Fig. 5. Bootstrap tree for ITS2 using the NJ method – Kimura 2-parameter distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale bar)

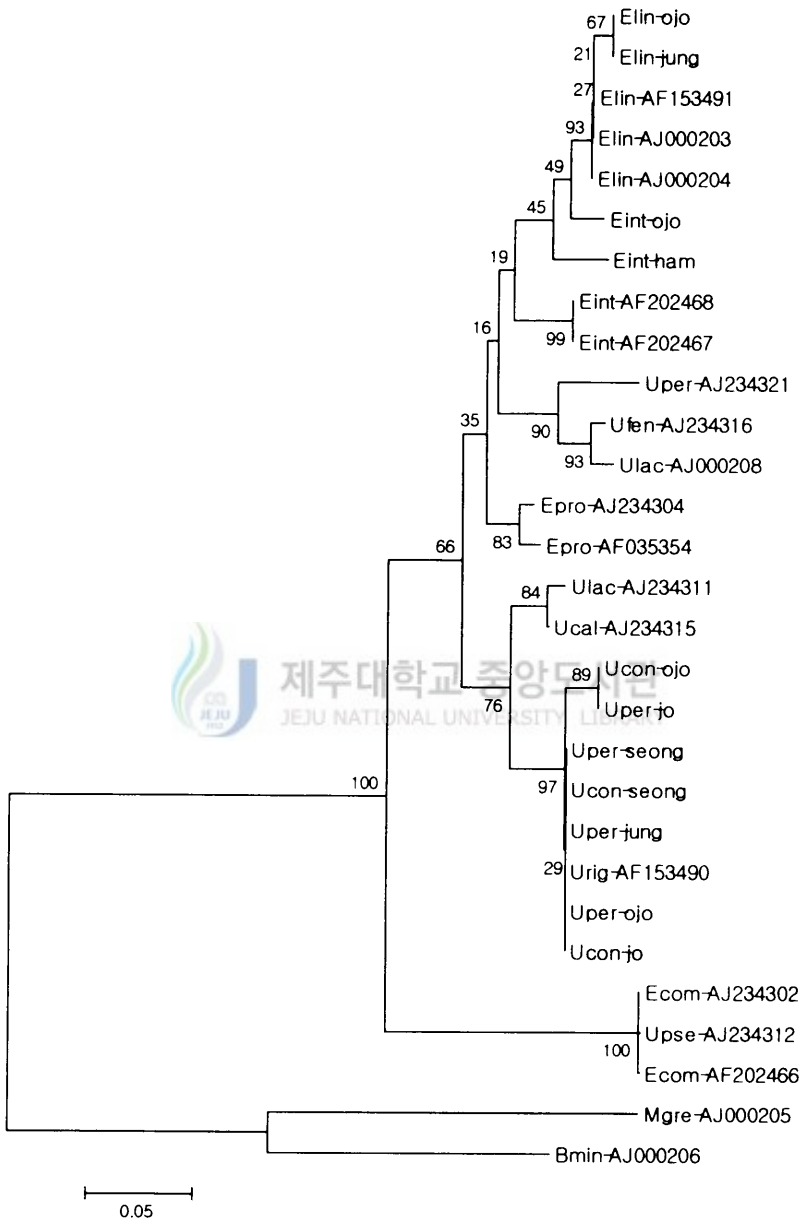


Fig. 6. Bootstrap tree for ITS2 using the NJ method – Tamura and Nei's distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale).

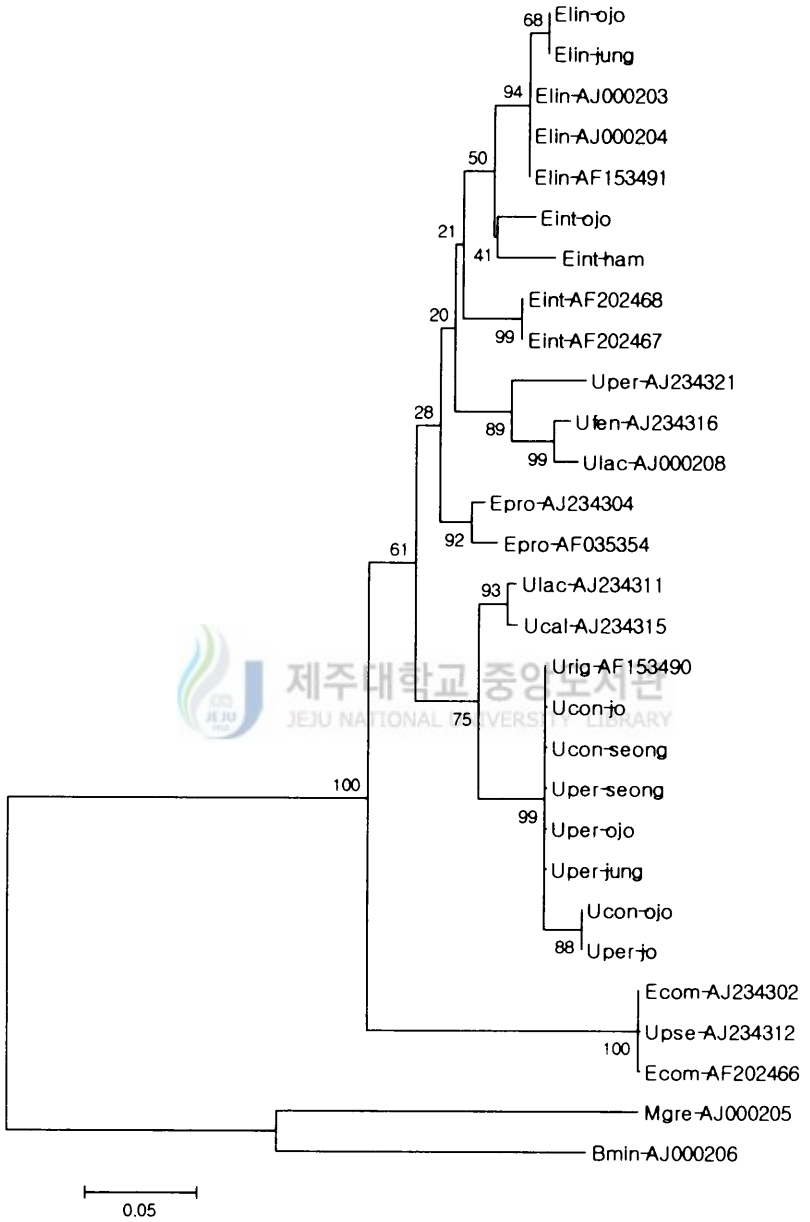


Fig. 7. Bootstrap tree for ITS2 using the ME method – Jukes and Cantors distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale).

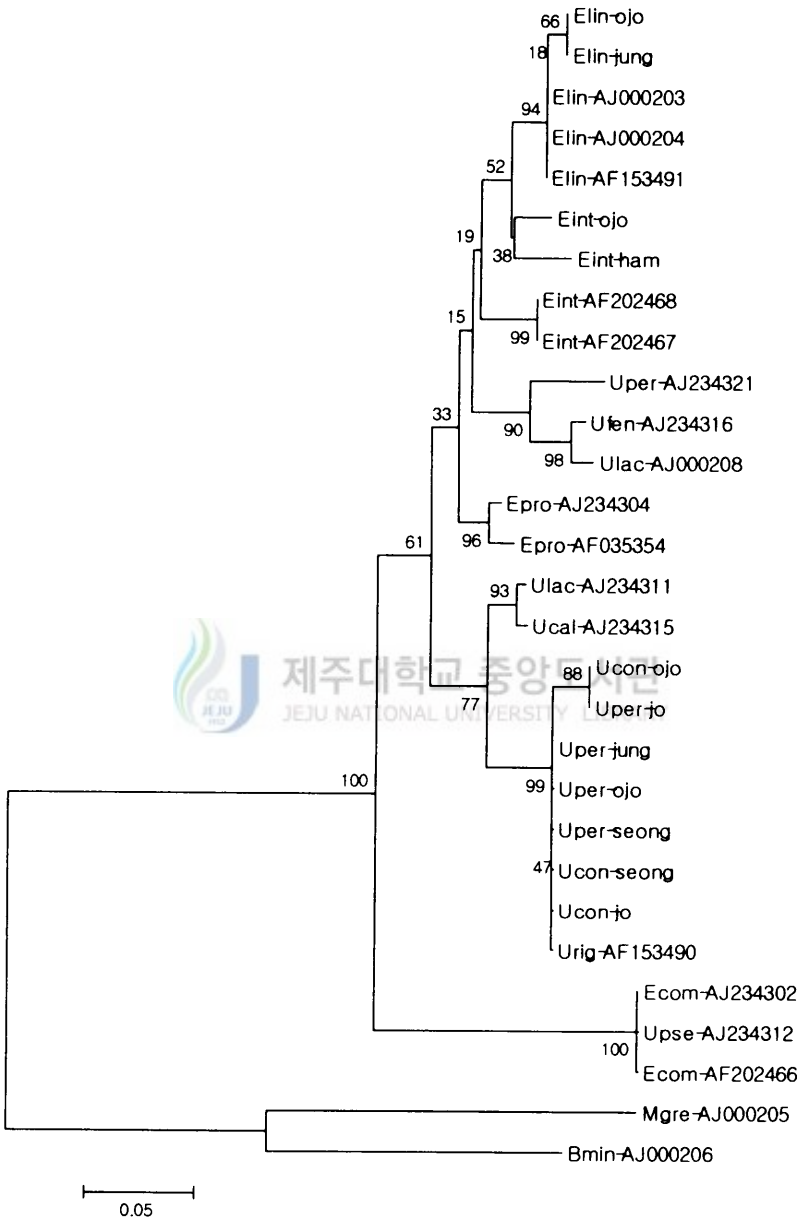


Fig. 8. Bootstrap tree for ITS2 using the ME method – Kimura 2-parameter distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale bar).

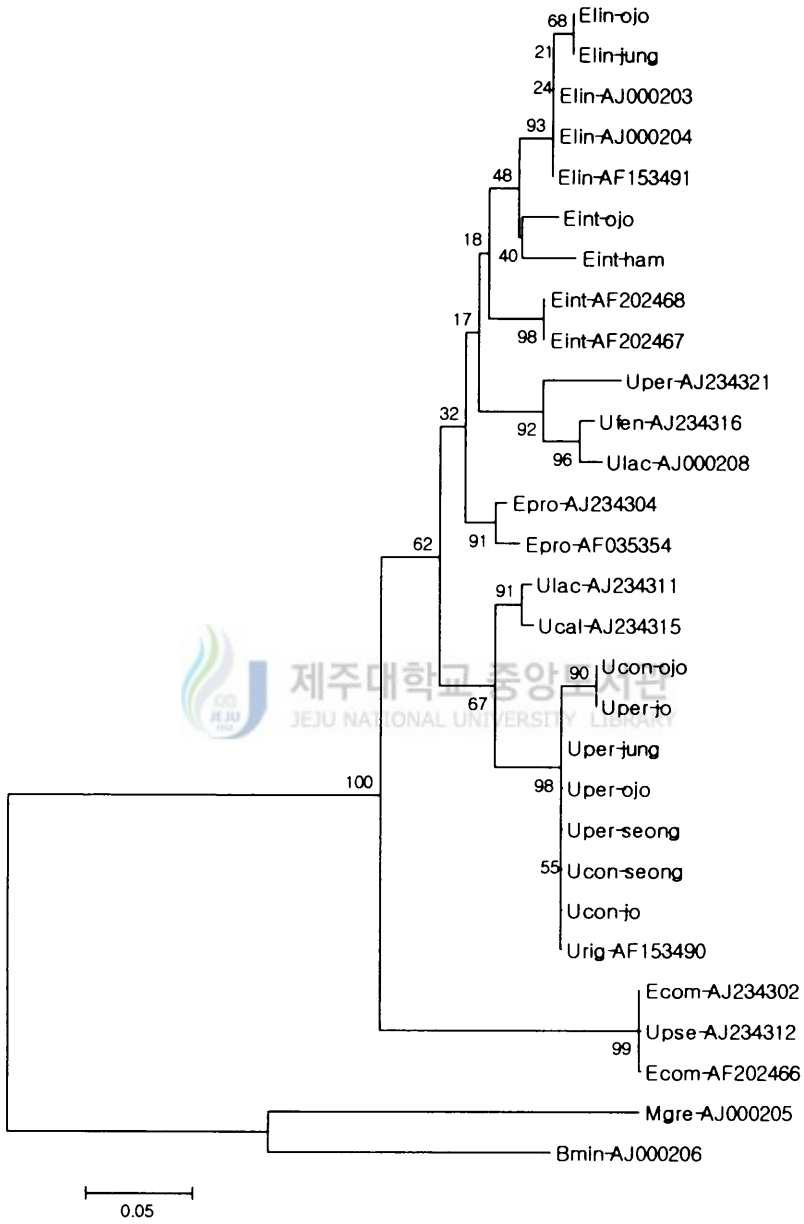


Fig. 9. Bootstrap tree for ITS2 using the ME method – Tamura and Nei's distance. Numbers on the nodes indicate bootstrap value (500 replicates). Branch lengths are proportional to the estimated mean number of substitutions site (see scale bar).

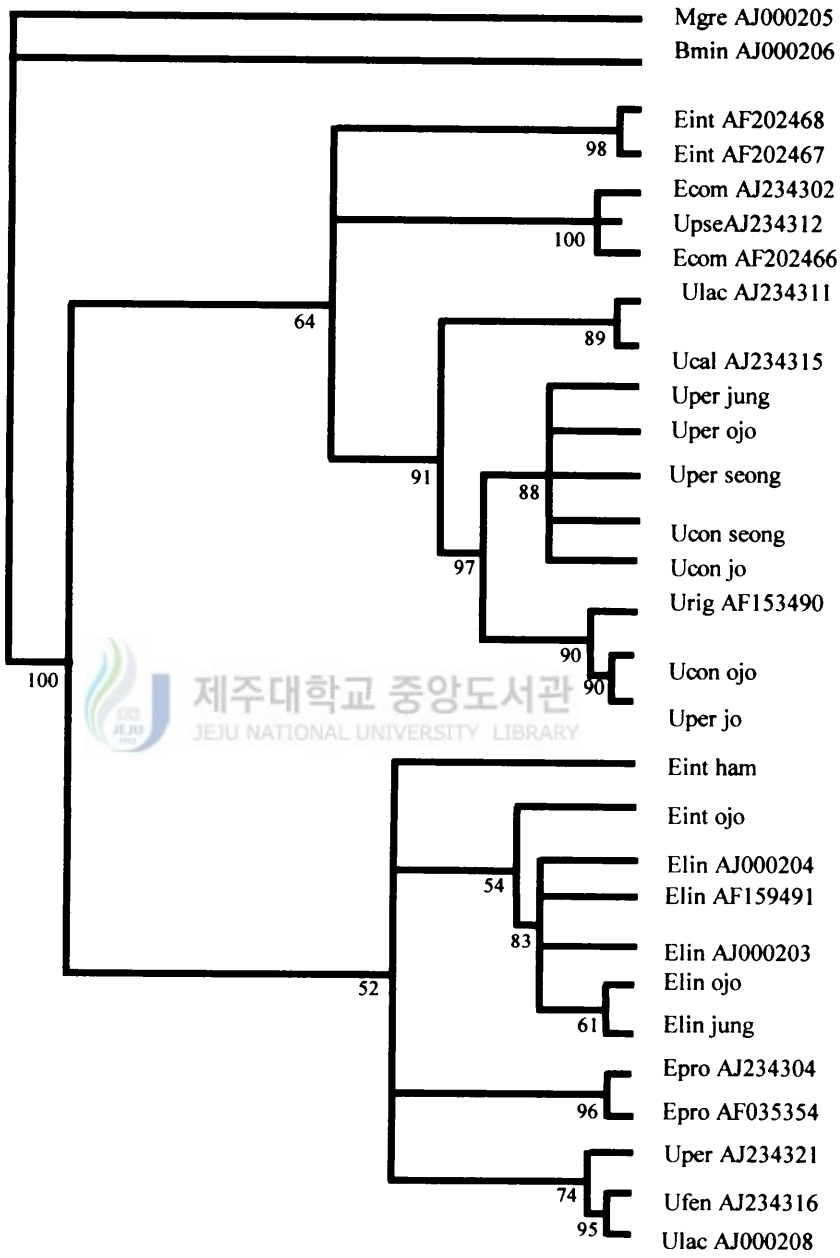



Fig. 10 MP tree for ITS2 (Tv:Ts = 3:1). Numbers on the nodes indicate bootstrap value (1000 replicates).




Fig. 11. MP tree for ITS2 (unweighted). Numbers on the nodes indicate bootstrap value (1000 replicates).

14.4%). Thus, those data indicate that there is no evolutionary distinction to be made between the members. This is not, however, suggesting that they are same species. Other samples showed a high level of divergence and bootstrap support for mixed clades containing representatives of respective species ranged from 74% to 100% (Fig. 10). Therefore, their low values of interspecific divergence were distinct phenomena in phylogenetic trees especially. All phylogenetic trees used *Blidingia nima* and *Monostroma grevillei* (Monostromataecae) as outgroup. They were placed in a well-supported clade while sequence divergence has a high level (from 43.1% to 55.8%).

# Ⅳ. Discussion

Recently, the ITS regions have been studied in several field especially molecular phylogenetic relationships (Leskinen and Pamilo, 1997; Pillmann et al., 1997; Rousseau et al., 2000; Tan et al., 1999; Stiger et al., 2000) and morphological analysis (Blomster et al., 1998, 1999; Coat et al., 1998; Malta et al., 1999; Fama et al., 2000; Woolcott et al., 2000) in algae. Because they vary to different degrees between taxonomic species, and their alignments have been used for phylogenetic purposes (Coleman and Mai, 1997). The general characteristics of the 18S – 28S intergenic region are similar to those in other organisms. The 5.8S rRNA gene sequence is conserved relative to the ITS sequences (Leskinen and Pamilo, 1997). The ITS sequences of *Enteromorpha* and *Ulva* are short in comparison to those found in most green algae, being most similar in length to the homologous sequence in *Acrosiphonia arcta* and some angiosperms (Bakker et al., 1992).



In this study, the length of ITS2 were ranged from 167 to 203 bp. G+C content values were higher than A and T. These values were similar to those of others published (Leskinen and Pamilo, 1997).

Two genera, *Enteromorpha* and *Ulva* are widely regarded as easily distinguishable because of their dramatically different morphologies: *Ulva* species are flat, lettucelike blades with two cell layers thickness, and *Enteromorpha* species form hollow liquid or gas-filled tubes with one cell thickness, which may also be highly branched. However, cell walls do not merely provide rigidity. They are essential to cell growth and developmental processes, such as axis formation in zygotes and branching in growing plants. When walls are too weak, development may be impossible, as in a mutant form of *Ulva* that grew as an aggregate of undifferentiated cells, rather than first forming a filament and later a holdfast plus blade (Lobban and Harrison, 1994). This flexibility of form among genetically homogeneous plants corroborates results of earlier culture studies that showed the development of both

tubular and bladelike thalli from the same zoospore populations of several *Ulva* species (Gayral, 1967; Bonneau et al., 1977; Provasoli and Pintner 1980). Gayral (1967) reported the occurrence of tubular thalli from both zoospore and gamete (parthenogenetic) cultures. The majority of both zoospores and parthenogenetic gametes developed into leafy thalli, whereas some developed into tubular ones. Culture study of Bonneau et al. (1997) showed progeny with some of the thalli were distromatic in some parts and they (Bonneau et al., 1997) questioned the validity of maintaining *Ulva* and *Enteromorpha* as two separate genera. Provasoli and Pintner (1980) showed that *Ulva* cultures could form uniseriate filaments when axenic or *Enteromorpha* like-tubes if grown with particular bacteria (Tan et al., 1999). Nakanishi et al. (1996) found that live bacteria are required for normal morphogenesis of *Ulva pertusa* in culture.

The results of this investigation showed that two genus *Ulva*, *Enteromorpha* grouped in a monophyletic assemblage with 100% bootstrap support in all phylogenetic trees. However, a thorough examination of these characters from representatives in this study does not provide data to identify any unique morphological features in all phylogenetic trees. Throughout analyzing ITS2 sequences in this study, it is proved that *U. conglobata* and *U. pertusa* assemblages were monophyletic groups even through morphological differences. It indicates that there is no evolutionary diversification to make them distinct. Recent studies reported that *Ulva* has a morphological variation especially in *U. rigida* (Phillps, 1984).

This study revealed that *U. conglobata* and *U. pertusa* belongs to one clade in phylogenetic tree. Also, *Enteromorpha* and *Ulva* are not distinct evolutionary entities, and can result in a plant with either a blade or a tube morphology as proposed by Tan et al. (1999). In the future, this study should be accompanied with developmental method and population translation relative with environmental factor to resolve many given question. This data could be applied to interspecific and population variation, together with fouling research in green algae.

# V. Summary

*Enteromorpha* and *Ulva* are ubiquitous and environmentally important genera. These members are tubular or membranous in gross morphology. Molecular sequence data were used to clarify the phylogenetic relationships of *Enteromorpha* and *Ulva*. Cloned internal transcribed spacer sequences (ITS2, flanking the 5.8S and 26S gene of the nuclear ribosomal genes) were aligned with sequences from genebank (18 samples), and subjected to neighbor joining, minimum evolution distance and parsimony analysis. Samples were collected from five localities in Jeju, including eleven purported individuals, four species within these two genera in the Ulvaceae.

In this study for Ulvaceae, the length of ITS2 varied between 167 and 203 bp. The results of this investigation showed that two genus, *Ulva and Enteromorpha* grouped in a monophyletic assemblage with 100% bootstrap support in all phylogenetic trees. However, a thorough examination of these characters from representatives in this study does not provided to identify any unique morphological features for clades in this tree.

This study revealed that *Ulva conglobata* and *Ulva pertusa* belongs to one clade in phylogenetic tree. Also, *Enteromorpha* and *Ulva* are not distinct evolutionary entities, and can result in a plant with either a blade or a tube morphology. This data, which was resulted from this investigation could be applied to interspecific and population variation, beside of fouling research in green algae.

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